Journal of Biomolecular NMR, 2 (1992) 227–233
ESCOM

J-Bio NMR 054

Positive φ-angles in proteins by nuclear magnetic resonance spectroscopy

Svend Ludvigsen* and Flemming M. Poulsen**

Carlsberg Laboratorium, Kemisk Afdeling, Gamle Carlsberg Vej 10, DK-2500 Valby, Copenhagen, Denmark

Received 1 August 1991
Accepted 3 December 1991

Keywords: Left-handed turns; Proteins; Positive φ-angles; 2D NMR; Barnase; Barwin; CI-2; Lysozyme

SUMMARY

Non-glycine residues with positive φ-angles have been identified in four proteins, barley serine proteinase inhibitor CI-2, bacterial ribonuclease (barnase) of *Bacillus amyloliquefaciens*, hen egg white lysozyme and a basic protein from barley seed (barwin) by use of nuclear magnetic resonance spectroscopy. By accurate measurements of the coupling constant $^3J_{H^NH^\alpha}$ and integration of the nuclear Overhauser H^N-H^α cross peak, positive φ-angles could be determined reliably to $60^\circ \pm 30^\circ$, in full agreement with the crystal structures for lysozyme, barnase and serine proteinase inhibitor CI-2. The work emphasizes that positive φ-angles can also occur in non-glycine residues and in the four proteins, positive φ-angles have been observed for the residue types aspartic acid, asparagine, arginine, serine, glutamine, histidine, tyrosine, tryptophan and phenylalanine. The measured $^3J_{H^NH^\alpha}$ coupling constants and the intensity of the intraresidue H^N-H^α NOEs agree well with the solution structures of three of the proteins, using the existing parametrization of the Karplus curve (Pardi, A., Billeter, M. and Wüthrich, K. (1984) *J. Mol. Biol.*, **180**, 741–751; Ludvigsen, S., Andersen, K.V. and Poulsen, F.M. (1991) *J. Mol. Biol.*, **217**, 731–736).

INTRODUCTION

Procedures for identification of the common secondary structure elements in protein structures using nuclear magnetic resonance spectroscopy have long been well established (Wüthrich, 1986). However, rare conformations do occur, and therefore, in structure studies it is necessary to ensure that such structure elements are not overseen. One such element is the amino acid residue with a positive φ-angle. Traditionally, it has been canonized that except for glycine residues the positive

* Present address: Novo Research Institute, Novo Nordisk, Novo Allé, DK-2880 Bagsværd, Copenhagen, Denmark.
** To whom correspondence should be addressed.

0925-2738/$ 5.00

φ-angle conformation is not allowed for amino acid residues in proteins. However, a recent investigation (Nicholson et al., 1989) indicates that non-glycine residues can exist with positive φ-angles in an energetically stable conformation. Overington et al. (1990) have examined a sample of high-resolution protein structures determined by X-ray crystallography and have thereby shown that apart from glycine representing 54% of all occurrences of positive φ-angles, asparagine represents 17%, aspartic acid 7% and serine 3%, leaving 19% to the remaining amino acid residue types. Overall, since positive φ-angles for non-glycine residues occur in approximately 2% of the cases in proteins (Wilmot and Thornton, 1990), it is likely that they exist in proteins of the size that NMR spectroscopy is most frequently applied to.

The structural characteristics of the positive φ-angles are a dihedral angle of 60° to 90° and a very short distance between H^N and H^α. These properties are determined by NMR as a coupling constant of approximately 6.8 Hz and a very intense NOE, respectively (Kline et al., 1988). The dihedral angle restraint boundaries that are typically set for a coupling constant of this size define only the negative range, and the distance restraint of a strong NOE is typically set with an upper boundary of 2.7 Å. As a result, structure calculations based on these restraints will result in a negative φ-angle between $-60°$ and $-90°$ and a minor violation of the distance restraint. Despite the fact that the inputs to the structure calculations are formally correct, positive φ-angles can therefore be overseen unless a full quantitative analysis is applied, that determines both the size of the $^3J_{H^N H^\alpha}$ and the size of the NOE.

In the present work we have examined the occurrence of positive φ-angles in four of the proteins that we have studied by NMR spectroscopy, using the quantitative method we proposed recently (Ludvigsen et al., 1991a). The proteins are barley serine proteinase inhibitor (CI-2), bacterial ribonuclease (barnase) of *Bacillus amyloliquefaciens*, hen egg white lysozyme and a basic protein from barley seed (barwin). The first three of these protein structures have been determined by X-ray crystallography, therefore we could compare directly the finding by NMR studies of positive φ-angles in solution structures with the results obtained in crystals.

METHODS

2QF-COSY (Piantini et al., 1982; Rance et al., 1983) and NOESY (Jeener et al., 1979; Anil-Kumar et al., 1980,1981) spectra of the four proteins CI-2, barnase, lysozyme and barwin were acquired at conditions according to Kjær et al. (1987), Redfield and Dobson (1988), Bycroft et al. (1990) and Ludvigsen and Poulsen (1992a). Each pair of COSY and NOESY (with mixing times of 150 ms) spectra was recorded under the same conditions and subsequently processed using the same apodization functions, typically sine-bells shifted $\pi/5$. The resolution in the ω_2 direction was for all four sets of spectra between 0.8 and 1.0 Hz. Coupling constants were measured using the method previously described relying on the coupling information present in both the antiphase cross peak in COSY and inphase cross peak in NOESY in the ω_2 direction of an H^N resonance (Ludvigsen et al., 1991a). The coupling constant $^3J_{H^N H^\alpha}$ extracted by respectively adding and subtracting the anti- and inphase peak trajectories is accurate apart from the imperfections caused by noise. Measurements with this method were accurate within 0.5 Hz, provided a signal-to-noise ratio better than 10:1 could be achieved. Intensities of the intraresidue NOE H^N-H^α cross peaks were measured using an algorithm capable of integrating partly overlapping cross peaks (Shen and Poulsen, 1990) and compared with average intensity of the intraresidue NOE H^N-H^α cross

peaks from residues in α-helices. Since the distance between H^N and H^α in a model α-helix is approximately 2.8 Å, this measured NOE intensity in experimental data sets can be used for calibration. The measurements of coupling constants and cross-peak integrations were carried out using the program PRONTO (Kjær et al., 1991).

The measurements of $^3J_{H^NH^\alpha}$ coupling constants and intensities of the intraresidue H^N-H^α NOESY cross peaks in the four proteins are summarized in Figs. 1 and 2. The accuracy of the integration of NOEs is better than 15% by comparing intensities of cross peaks on each side of the diagonal in the NOESY spectrum, whereas the coupling constants are accurate within 0.5 Hz.

For lysozyme H^N-H^α NOESY cross peaks in the middle of the α-helix extending from Ile^{88} to Asp^{101} were selected as representatives of typical α-helix members in the crystal structure of lysozyme (Handoll, 1985). Thus H^N-H^α NOESY cross-peak intensities were normalized to the average intensities of residues Ala^{90}, Ser^{91}, Val^{92}, Cys^{94}, Lys^{96} and Ile^{98}, for which in all cases reliable integrations could be performed. In the case of barnase, intensities were normalized relative to the average H^N-H^α NOESY cross-peak intensities of Phe^7, Asp^8, Val^{10}, Ala^{11}, Tyr^{13}, Leu^{14} and Gln^{15} of the N-terminal α-helix, which is well defined in the crystal structure (Mauguen et al., 1982) and in the solution structure (Bycroft et al., 1991). For CI-2 structures (Clore et al., 1987; McPhalen and James, 1987; Ludvigsen et al., 1991b) the residues in the well-defined α-helix from residues 32-40 were used for normalization. The solution structure of barwin (Ludvigsen and Poulsen, 1992b) has a well-defined α-helix from residues Leu^{40} to Tyr^{46}, which were used for calibration. The chemical shifts of all the relevant NOESY and COSY cross peaks in the spectra were obtained from the lists of published assignments (Kjær et al., 1987; Redfield and Dobson, 1988; Bycroft et al., 1990; Ludvigsen and Poulsen, 1992a).

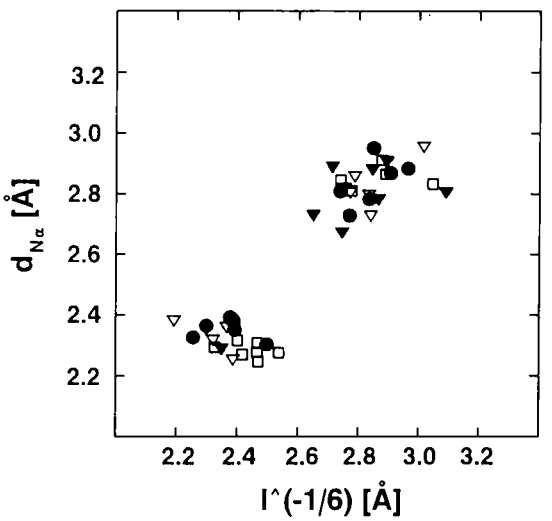

Fig. 1. Comparison of the H^N-H^α distances ($d_{N\alpha}$) and NOE intensities in α-helical residues and residues with positive φ-angles. For each protein the NOE intensities have been normalized to the average H^N-H^α distance in the reference α-helical residues; barnase (●), barwin (▽), CI-2 (▼) and lysozyme (□).

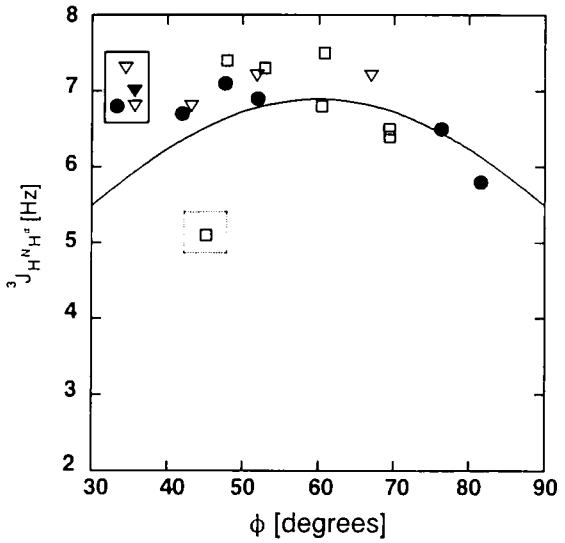

Fig. 2. Correlation between measured coupling constants and positive φ-angles as determined in solution for barnase, barwin, and CI-2, and in crystal for lysozyme. The curve is the parametrization of the Karplus function based on the crystal structure of BPTI. The upper rectangle frames 4 measurements of residues where the φ-angle was poorly determined (His^{102} in barnase, Asp^5 and Ser^{28} in barwin, and Asp^{74} in CI-2). The coupling constant of 5.1 also boxed was measured for Gln^{57} in lysozyme. Legend as in Fig. 1.

RESULTS AND DISCUSSION

The examination of the H^N-H^α NOE intensities measured for residues with positive φ-angles relative to those of residues in α-helices is shown in Fig. 1. The diagram clearly shows the separation between the residues with positive φ-angles and those of α-helical residues and suggests that positive φ-angles could indeed be identified simply on the basis of a comparison of these NOE intensities. In terms of distances the smallest difference between the two categories of conformations is in the range between 0.20 and 0.37 Å for the four proteins and these differences are in all four cases significant. In the study of lysozyme the comparison of the intensities showed that the ratio between the two groups of NOEs was somewhat smaller than seen in the other three proteins.

Similarly we have compared the correlation between the measured coupling constants and positive φ-angles observed in the structures determined in solution by NMR for barnase, CI-2 and barwin and in the structure of lysozyme in crystals (Fig. 2). The residues with positive φ-angles are seen to have coupling constants close to the value of 7 Hz found in the parametrization of the Karplus curve (Karplus, 1959) determined by Pardi et al. (1984) by comparison to a crystal structure of the basic pancreatic trypsin inhibitor, BPTI. Thus, with a large number of experimental data for residues with positive φ-angles the existing calibration of the Karplus curve seems to be quite reasonable. Some of the measured $^3J_{H^NH^\alpha}$ coupling constants reported here were previously used for a calibration of the Karplus curve using the φ-angles measured in crystal structures of CI-2 and barnase (Ludvigsen et al., 1991a) where it was shown that the resulting Karplus curve dif-

fered only little from the parameters found by Pardi et al. (1984). Such calibrations are not necessarily accurate because φ-angles in crystal structures and in solution structures may not be the same, and the φ-angles in crystal and solution structures are determined with an accuracy of $\pm 10^{\circ}$ or more. Given that only a few positive φ-angles have been included in the calibration of the Karplus curve, it seemed relevant to report these measured $^{3}J_{H^{N}H^{\alpha}}$ coupling constants of residues with positive φ-angles.

The coupling constant $^{3}J_{H^{N}H^{\alpha}}$ alone cannot be used to determine positive φ-angles due to the Karplus function and although the results here have shown that positive φ-angles may indeed be determined just from measurements of the H^{N}-H^{α} NOE intensities we would still advice that the criteria for the size of the coupling constant being around 7 Hz should be applied as well to ensure unambiguous identification.

The existence of positive φ-angles is mostly associated with the presence of turns in proteins. Turns consist of four residues, and the classification of turns (Venkatachalam, 1968) is based on φ- and ψ-angles of residues 2 and 3 (Fig. 3). These classifications can be translated into measurable NOEs and $^{3}J_{H^{N}H^{\alpha}}$ coupling constants (Wüthrich, 1986) appropriate for NMR investigations. The differentiation between type I and III, I′ and III′ is impractical since the definitions of turns allow a deviation of 40° for the φ- and ψ-angles. A recent proposal for a nomenclature of turns in proteins does not distinguish between type I and III, I′ and III′ turns (Wilmot and Thornton, 1990).

The identification of positive φ-angles is a prerequisite for the subsequent identification of reverse turns of type I′, II and II′, and (III′). The left-handed turns of type I′ (and type III′) have two consecutive residues with positive φ-angles and as such they are readily determined. In general,

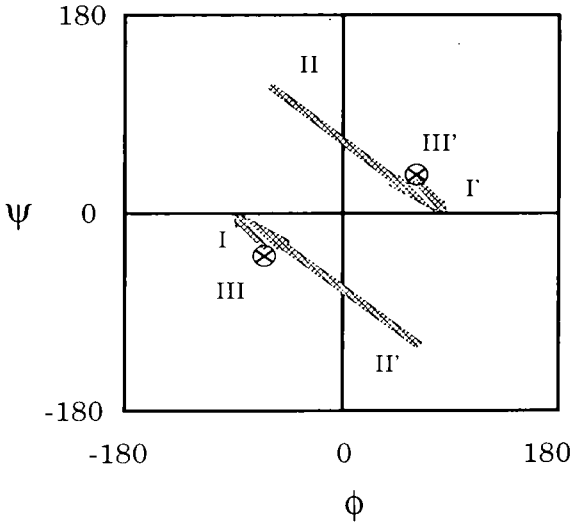

Fig. 3. Ramachandran plot defining the 3 turns and their 'mirrors'. The names of the turns are given according to Venkatachalam (1968). A turn consists of 4 residues, and the arrow shows the position in the φ,ψ plot from residue 2 to 3. Two identical positions for residues 2 and 3 in type III and III′ turn are indicated by a ⊕. Turns are further characterized by a hydrogen bond from H^{N} in residue 4 to O in residue 1.

however, it was of interest to examine the structures determined by NMR spectroscopy for turn configurations around the residues with identified positive φ-angles. For the solution structures this analysis could be performed unbiased because in the structure calculations we have used relatively broad torsion-angle-restraint limits of structures of barnase (60° ± 50°) and barwin (70° ± 40°), and a somewhat narrower limit for CI-2 (60° ± 20°). None of these restraints was violated in the structures, however, in Asp^{5} and Ser^{28} in barwin and in Asp^{74} in CI-2 the φ-angles were close to the lower restraint boundary (see Fig. 2). Furthermore, hydrogen-bond restraints were not applied for turns identified in the secondary structure evaluation. Therefore, for the structures of CI-2, barnase and barwin the presence of regular turns was examined in the calculated structures as judged by the φ- and ψ-angles in residues 2 and 3 and the presence of a hydrogen bond between residues 1 and 4. This showed that a number of residues with positive φ-angles was not in any regular type of turn, and, in particular, the existence of left-handed turns in each of the solution structures of barnase and barwin at the sites where two consecutive residues had positive φ-angles. Overall, the sites with positive φ-angles seen in the solution structures were also found in the crystal structures, and the turns associated with these were in full agreement with respect to type. Even the sites where no regular turn type was associated were the same in crystal and solution. The latter observation emphasizes that the presence of positive φ-angles should not be used as a single criterion for the presence of a turn. In barnase, positive φ-angles were observed for His^{18}, Asn^{58}, Asn^{77}, Trp^{94}, His^{102} and Tyr^{103}; in barwin for Asp^{5}, Asn^{19}, Trp^{20}, Ser^{28} and Arg^{124}; in CI-2 for Asp^{74}; and in lysozyme for Asn^{19}, Arg^{21}, Asn^{37}, Phe^{38}, Gln^{57}, Asn^{74} and Asn^{77}. The coupling constants measured for lysozyme were in full agreement with those determined by Smith et al. (1991).

Positive φ-angles for non-glycine residues have previously been reported (Crawford et al., 1973; Lewis et al., 1973) in various proteins for which high-resolution crystal structures were available. The present work has focused on the occurrence of positive φ-angles in non-glycine residues as determined by NMR spectroscopy. Although it is well known that non-glycine residues can adopt positive φ-angles in crystals, only very few cases have been observed in solution structures so far determined by NMR spectroscopy (Kline et al., 1988; Clore et al., 1991). The present work has demonstrated that positive φ-angles in non-glycine residues, present in crystal structures of proteins do also exist in solution and the existence of these can be determined unambiguously by NMR. Furthermore it has also been the purpose of this paper to emphasize that although positive φ-angles are relatively common for residue types like asparagine, aspartic acid and serine, their occurrence is not limited to these types of residues. In fact the present work has involved the determination of positive φ-angles in residues such as arginine, glutamine, histidine, tyrosine, tryptophan and phenylalanine. In this respect the present paper may also serve as a reminder that these rare conformations do exist in proteins despite the claims, once, that these conformations are energetically forbidden.

ACKNOWLEDGEMENTS

Pia Mikkelsen is thanked for skilled preparation of NMR samples. Kim V. Andersen and Mogens Kjær have coded the coupling-constant measurement routine into the program Pronto (Kjær et al., 1991). This paper is a contribution from the Danish Protein Engineering Research Centre (PERC).

REFERENCES

Anil-Kumar, Ernst, R.R. and Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun.*, **95**, 1–6.

Anil-Kumar, Wagner, G., Ernst, R.R. and Wüthrich, K. (1981) *J. Am. Chem. Soc.*, **103**, 3654–3658.

Bycroft, M., Sheppard, R.N., Lau, S.T.K. and Fersht, A.R. (1990) *Biochemistry*, **29**, 7425–7432.

Bycroft, M., Ludvigsen, S., Fersht, A.R. and Poulsen, F.M. (1991) *Biochemistry*, **30**, 8697–8701.

Clore, G.M., Gronenborn, A.M., Kjær, M. and Poulsen, F.M. (1987) *Prot. Eng.*, **1**, 305–311.

Clore, G.M., Wingfield, P.T. and Gronenborn, A.M. (1991) *Biochemistry*, **30**, 2315–2323.

Crawford, J.L., Lipscomb, W.N. and Schellman, C.G. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 538–542.

Handoll (1985) Ph. D. Thesis, Oxford University.

Jeener, J., Meier, B.H., Bachmann, P. and Ernst, R.R. (1979) *J. Chem. Phys.*, **71**, 4546–4553.

Karplus, M. (1959) *J. Phys. Chem.*, **30**, 11–15.

Kjær, M., Ludvigsen, S., Sørensen, O.W., Denys, L.A., Kindtler, J. and Poulsen, F.M. (1987) *Carlsberg Res. Commun.*, **52**, 327–354.

Kjær, M., Andersen, K.V., Ludvigsen, S., Shen, H., Windekilde, D., Sørensen, B. and Poulsen, F.M. (1991) In *Computational Aspects of the Study of Biological Macromolecules* (Eds, Hoch, J.C., Poulsen, F.M. and Redfield, C.) NATO ASI Series, *Ser. A*, **225**, 291–302.

Kline, A.D., Braun, W. and Wüthrich, K. (1988) *J. Mol. Biol.*, **204**, 675–724.

Lewis, P.N., Momany, F.A. and Scheraga, H.A. (1973) *Biochim. Biophys. Acta*, **303**, 211–229.

Ludvigsen, S., Andersen, K.V. and Poulsen, F.M. (1991a) *J. Mol. Biol.*, **217**, 731–736.

Ludvigsen, S., Shen, H., Kjær, M., Madsen, J.C. and Poulsen, F.M. (1991b) *J. Mol. Biol.*, **222**, 621–635.

Ludvigsen, S. and Poulsen, F.M. (1992a), submitted for publication.

Ludvigsen, S. and Poulsen, F.M. (1992b), submitted for publication.

Mauguen, Y., Hartley, R.W., Dodson, E.J., Dodson, G.G., Bricogne, G., Chothia, C. and Jack, A. (1982) *Nature*, **297**, 162–164.

McPhalen, C.A. and James, M.N.G. (1987) *Biochemistry*, **26**, 261–269.

Nicholson, H., Söderlind, E., Tronrud, D.E. and Matthews, B.W. (1989) *J. Mol. Biol.*, **210**, 181–193.

Overington, J., Johnson, M.S., Šali, A. and Blundell, T.L. (1990) *Proc. Roy. Soc. Lond. B.*, **241**, 132–145.

Pardi, A., Billeter, M. and Wüthrich, K. (1984) *J. Mol. Biol.*, **180**, 741–751.

Piantini, U., Sørensen, O.W. and Ernst, R.R. (1982) *J. Am. Chem. Soc.*, **104**, 6800–6801.

Rance, M., Sørensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **117**, 479–485.

Redfield, C. and Dobson, C.M. (1988) *Biochemistry*, **27**, 122–136.

Shen, H. and Poulsen, F.M. (1990) *J. Magn. Reson.*, **89**, 585–594.

Smith, L.J., Sutcliffe, M.J., Redfield, C. and Dobson, C.M. (1991) *Biochemistry*, **30**, 986–996.

Venkatachalam, C.M. (1968) *Biopolymers*, **6**, 1425–1436.

Wilmot, C.M. and Thornton, J.M. (1990) *Prot. Eng.*, **3**, 479–493.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, New York.